Tsetse immune responses and trypanosome transmission: Implications for the development of tsetse-based strategies to reduce trypanosomiasis

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Tsetse flies are the medically and agriculturally important vectors of African trypanosomes. Information on the molecular and biochemical nature of the tsetse/trypanosome interaction is lacking. Here we describe three antimicrobial peptide genes, *attacin, defensin*, and *diptericin*, from tsetse fat body tissue obtained by subtractive cloning after immune stimulation with *Escherichia coli* and trypanosomes. Differential regulation of these genes shows the tsetse immune system can discriminate not only between molecular signals specific for bacteria and trypanosome infections but also between different life stages of trypanosomes. The presence of trypanosomes either in the hemolymph or in the gut early in the infection process does not induce transcription of *attacin* and *defensin* significantly. After parasite establishment in the gut, however, both antimicrobial genes are expressed at high levels in the fat body, apparently not affecting the viability of parasites in the midgut. Unlike other insect immune systems, the antimicrobial peptide gene *diptericin* is constitutively expressed in both fat body and gut tissue of normal and immune stimulated flies, possibly reflecting tsetse immune responses to the multiple Gram-negative symbionts it naturally harbors. When flies were immune stimulated with bacteria before receiving a trypanosome containing bloodmeal, their ability to establish infections was severely blocked, indicating that up-regulation of some immune responsive genes early in infection can act to block parasite transmission. The results are discussed in relation to transgenic approaches proposed for modulating vector competence in tsetse.

Glossina | insect immunity | vector control | transgenesis | symbiosis

The life cycle of the parasitic African trypanosomes (Euglenozoa: Kinetoplastida) in their insect vector, the tsetse fly (Diptera: Glossinidae), begins when it feeds from an infected mammalian host. For successful transmission, the parasite undergoes two stages of differentiation in the fly: first, establishment in midgut and then maturation in the mouthparts or salivary glands. In the midgut, the mammalian bloodstream parasites rapidly differentiate to procyclic forms and begin to replicate (establishment). Once established in the midgut, trypanosomes migrate forwards to the proventriculus and the mouthparts, where they begin to differentiate into epimastigotes and eventually colonize the proboscis or salivary glands, depending on the parasite species (1). Here they differentiate into metacyclic forms infective to mammals (maturation) and can be transmitted to the next host during blood feeding by the fly (2). It is generally thought that during normal development in the fly, there are no intracellular stages, although reports of intracellular *Trypanosoma brucei rhodesiense* (3, 4) and *Trypanosoma congolense* (5) in the anterior midgut cells have been published. It is also thought that during normal infection, trypanosomes do not cross an epithelial barrier to enter the fly, although there are several reports of trypanosomes in the hemolymph of flies (6, 7).

Tsetse flies are in general refractory to parasite transmission, although little is known about the molecular basis for refractoriness. In laboratory infections, transmission rates vary between 1 and 20%, depending on the fly species and parasite strain (8–10), whereas in the field, infection with *T. brucei* spp. complex trypanosomes is typically detected in less than 1–5% of the fly population (11–13). Many factors, including lectin levels in the gut at the time of parasite uptake, fly species, sex, age, and symbiotic associations in the tsetse fly, apparently play a part in determining the success or failure of parasite infections (14). Tsetse flies have been shown to possess midgut lectin(s) that are capable of killing trypanosomes *in vivo* by a process resembling programmed cell death (14), and there is also indirect evidence to suggest that trypanosomes may be killed by an innate immune response in the fly (15, 16). Although there is some information demonstrating antimicrobial activity (17–19), the prophenoloxidase cascade (20), lectin (21), and hemocyte types (22), no information is available on tsetse innate defense mechanisms at molecular and biochemical levels. In addition to transmitting trypanosomes, tsetse flies also harbor multiple symbionts and rely on these associations for nutrition and fecundity (23). The two gut symbionts, *Sodalis glossinidius* and *Wigglesworthia glossinidia*, are Gram-negative bacteria closely related to *Escherichia coli*. How the trypanosomes and multiple symbionts can evade the natural immune mechanisms of tsetse is at present unknown.

This is the first report, to our knowledge, on the molecular characterization of immune responsive genes from tsetse. By using a suppression subtractive hybridization approach, cDNA fragments from *Glossina morsitans morsitans* were isolated from fat body after immune challenge with *E. coli* and procyclic trypanosomes. We present data on the expression profiles of the three antimicrobial peptide genes selected in this analysis, *defensin*, *attacin*, and *diptericin*, after challenge with bacteria and trypanosomes by both microinjection and direct feeding approaches. The regulation of immune peptide gene transcription in fat body was also studied in flies, which had established parasite infections in the gut. In addition, the ability of tsetse immune mechanism(s) to interfere with the establishment of parasite gut infections was investigated by stimulating tsetse

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Abbreviation: LPS, lipopolysaccharide.

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immune response(s) before delivering an infectious bloodmeal. The potential to modulate the vector competence via transgenesis is discussed in light of the role tsetse innate immune responses play in trypanosome establishment.

Materials and Methods

Tsetse. The *G. m. morsitans* colony at Bristol University was originally established from puparia collected in Zimbabwe. The colony maintained in the insectary at Yale University was established from puparia obtained in 1994 from the Bristol Tsetse Research Laboratory. Flies were maintained at 24 ± 1°C with 55–60% relative humidity and received defibrinated bovine blood at Yale and horse and pig blood at Bristol every other day by using an artificial membrane system (24).

Immune Stimulation. Adult nonteneral *G. m. morsitans* (4–6 days old) were microinjected through the wing base area of the pteropleuron with 2 μl of a mixture of live *E. coli* K12 RM148 at OD_{600} 0.4 in PBS containing 1×10^4 procyclics of *T. congolense* and *T. brucei*. For expression analysis, *T. b. rhodesiense* (strain Ytat 1.1) cells were used for immune challenge.

cDNA Subtraction and Construction of Enriched Libraries. The CLONTECH PCR-select cDNA subtraction kit was used to obtain the differentially expressed transcripts by a suppression subtractive hybridization method. For "tester" cDNA preparation, fat bodies were dissected from the abdomens of 50 flies 6–18 h after immune challenge, and mRNA was prepared by using Dynabeads Oligo $(dT)_{25}$ (Dynal, Great Neck, NY). "Driver" cDNA was produced from mRNA similarly extracted from fat bodies of unstimulated *G. m. morsitans* flies 6–18 h after their last bloodmeal. A subtracted library was prepared corresponding to an immune-challenge-induced mRNA pool. Similarly, a library was prepared corresponding to the reverse-subtracted mRNA pool, which represented suppressed transcripts. The subtracted pools were cloned by using the TA Cloning Kit (Invitrogen). A total of 102 randomly picked clones were studied by DNA sequencing analysis. Sequence homology searches of public databases were performed on the National Center for Biotechnology Information WWW server with the BLAST programs.

Cloning and Sequencing of Attacin, Defensin, and Diptericin cDNAs. To obtain full length cDNAs, a cDNA library was constructed from immune-induced fat body mRNA in the λZAPII cloning vector by using the ZAP-cDNA Synthesis Kit (Stratagene) according to the manufacturer's instructions. The library was estimated to contain a total of 2.3×10^6 independent clones. Partial attacin and defensin cDNA fragments were PCR-amplified with specific oligonucleotide primers: attacin (forward, 5′-GCACAGTATCATCTAACC-3′, and reverse, 5′-GCCAAGAGTATTCATATCG-3′); defensin (forward, 5′-CTTACACTATGTGCTGTTGTCG-3′, and reverse, 5′-GTGCAATAGCATACACCAC-3′). The PCR amplification conditions were 94°C for 3 min, 30 cycles of 30 sec at 94°C, 1 min at 55°C, and 1 min at 72°C in an MJ Research (Cambridge, MA) PTC-200 thermocycler. The amplification products were radiolabeled by random primer labeling to screen the library, and the purified cDNAs were characterized by DNA sequencing analysis. Comparative sequence analysis was carried out by using DNASTAR software programs (Lasergene, Madison, WI), SIGNALP Ver. 1.1 (Center for Biological Sequence Analysis, Technical University of Denmark, http://genome.cbs.dtu.dk/htbin/nph), and PSORT II analysis (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences, http://psort.nibb.ac.jp).

Expression Analysis in Response to Immune Challenge. Three groups of 40 flies each were microinjected with either 2 μl of PBS or live *E. coli* K12 XL-1 blue cells in PBS (OD_{600} 0.6) or 1×10^5 procyclic trypanosomes, respectively. Ten flies from each group were dissected at 6, 18, 30, and 48 h after microinjection, respectively, and fat body was collected. Fat body was also collected from a control (unstimulated) group of flies 12 h after their last bloodmeal. Similarly, three groups of 1-week-old flies received bloodmeals containing 1×10^5 cells/ml of cryopreserved bloodstream or procyclic trypanosomes or *E. coli*, respectively. Fat body was dissected and pooled from six flies in each group 8 and 24 h after the bloodmeals were administered. Fat body from control (unstimulated) flies was similarly analyzed 8 and 24 h after a regular bloodmeal. Total RNA (20 μg per lane) was electrophoresed on 2% formaldehyde agarose gels and transferred to nylon membranes (Hybond, Amersham Pharmacia). The *attacin* and *defensin* specific hybridization probes were prepared as described, and an RNA probe was generated for *diptericin* by using the RNA Labeling Kit (Amersham Pharmacia, catalogue no. RPN 3100). Hybridization conditions were as described. The findings were confirmed by three separate experiments.

***In Vitro* Antibacterial Assay.** Antibacterial assay with the synthetic diptericin 82-mer peptide was performed in sterile 96-well plates with a final volume of 100 μl, as described (25). Briefly, 90 μl of a suspension of a midlogarithmic phase cultures of *Sodalis*, *E. coli*, or procyclic *T. b. rhodesiense* was added to 10 μl of serially diluted diptericin peptide in sterile water. The final peptide concentrations ranged from 0.1 to 10 μM. Plates were incubated at 28°C for *Sodalis* and trypanosomes and at 30°C for *E. coli* with gentle shaking. Growth inhibition was measured by recording the increase of the absorbance at 600 nm for *E. coli*. Cell numbers were determined for *Sodalis* and trypanosomes over 48 h. The IC_{50} values correspond to a peptide concentration resulting in half of maximum absorption compared with normal bacterial growth.

Immune Regulation in Parasite-Infected Flies. To investigate gene expression during the course of parasite establishment, flies were given a procyclic trypanosome containing bloodmeal, and the fat body was dissected from groups of six flies after 3 and 6 days, respectively. On day 10, the rest of the flies were dissected and scored for gut parasite infections, and tissues were collected from infected (+) and uninfected (−) flies. The ability of parasite-infected flies to elicit an immune response was studied by Northern analysis. A group of 50 teneral adults were given a trypanosome containing bloodmeal supplemented with 0.015 M D+glucosamine prepared in saline to permit higher infection rates (26). After 20 days, half the flies were challenged with *E. coli* microinjection, whereas the other half were untreated. All flies were dissected after 24 h and microscopically examined for parasite infections. As expected for this colony, the infection prevalence was about 40%. Fat body for Northern analysis was collected from five individuals representing the four groups: parasite infected (+), parasite infected and immune stimulated (+I), parasite uninfected (−), and parasite uninfected and immune stimulated (−I).

Immune Stimulation and Trypanosome Establishment. Groups of teneral flies 24 h after emergence were either mock-injected with PBS or microinjected with live *E. coli* or with lipopolysaccharide (LPS) from *E. coli* J5 (Sigma) prepared in PBS at 1 mg/ml, respectively. A fourth group was untreated as control. Twenty hours after immune stimulation, flies were given one infectious bloodmeal containing procyclic trypanosomes and 0.015 M D+glucosamine. Flies that did not feed on the infectious bloodmeal were discarded, and all flies were subsequently maintained

on defibrinated sterile blood. On day 20, the prevalence of parasite infections in gut tissue was microscopically evaluated. Four independent replicates were done for the control and *E. coli* groups and three replicates for the PBS and LPS groups. After conducting an arcsine transformation on the proportional infection data, a single factor analysis of variance was performed. The analysis revealed there were no significant differences in infection prevalence between replicates ($F = 0.11$, $P = 0.95$), affirming the reliability of the experimental procedures. Thus, replicates were pooled for analysis according to treatment. Significant differences in the prevalence of infection among treatments were evaluated by χ^2 analysis. The infection prevalence in the control group served as the expected data against which infection prevalences in the *E. coli*, PBS, and LPS groups were tested. Statistical analysis was done by using SYSTAT, and differences were considered significant at $P < 0.05$.

Results

Characteristics of Antimicrobial Peptide cDNAs. In the suppression subtractive hybridization analysis, 35 of the 102 selected cDNA fragments were identified as known antimicrobial peptide genes, 12 corresponded to attacin, 9 to defensin, and 14 to diptericin homologues. As insect antimicrobial peptide genes often form families, the genes characterized here have been named *GmAttA*, *GmDefA*, and *GmDipA* to indicate that they are the first characterized sequences in these families in tsetse. For further molecular analysis, full length attacin (GmAttA), defensin (GmDefA), and diptericin (GmDipA) cDNAs were isolated.

The 845-bp *GmAttA* encodes a 208-aa putative peptide displaying 55% identity to *Drosophila melanogaster attacinA* and *B*, and 49 and 38% to *attacinC* and *D* gene products, respectively (Fig. 1*A*). The *GmAttA* ORF encodes a 5′-end 20-aa hydrophobic region with signal peptide characteristics indicating that it is secreted into the hemolymph. Analysis of three independent cDNA clones showed that unlike *Drosophila AttA*, *B*, and *C* gene products, the putative *GmAttA* peptide lacks a propeptide (activation) domain including the proteolytic cleavage site following the signal peptide. To confirm this finding, we used the fat body cDNA library as template to PCR-amplify *GmAttA*-specific fragments spanning the propeptide domain. The cloned and sequenced amplification products all indicated the absence of this domain (data not shown). The *attD* of *Drosophila* similarly lacks the propeptide domain, although it also lacks the signal peptide and is thought to be cytoplasmic in nature (27). The 188-aa mature *GmAttA* peptide has the N-terminal domain and the two glycine rich G-domains typically associated with the C-terminal region of attacins. Genomic PCR results with *GmAttA*-specific primers have shown that the N-terminal and G1 domain is separated by a 60-bp intervening sequence similar to the *Drosophila* attacin genes. It remains to be seen whether attacins also represent a gene family in tsetse with possibly different molecular characteristics.

The 457-bp *GmDefA* cDNA encodes an 87-aa preprodefensin peptide. A 49-bp 5′-untranslated region is followed by a putative hydrophobic signal of 19-aa sequence ending at Ala_{19}. On the basis of its alignment with other defensin peptides, the propeptide (34 aa) is further cleaved to the mature defensin (33 aa) (Fig. 1*B*). It has an arginine residue at base 54, which is found conserved in different defensins and may be involved in the proteolytic processing of the mature peptide. The putative GmDefA has the six conserved disulphide-paired cysteine residues found in insect defensins. The calculated molecular mass of prodefensin is 7,582 Da and of mature defensin is 3,600 Da. The potential isoelectric point of the mature GmDefA is 8.3, which suggests it has the cationic properties proposed for defensins (28).

The partial *GmDipA* cDNA product was 257 bp and encoded a 76-aa peptide lacking the beginning of its signal peptide

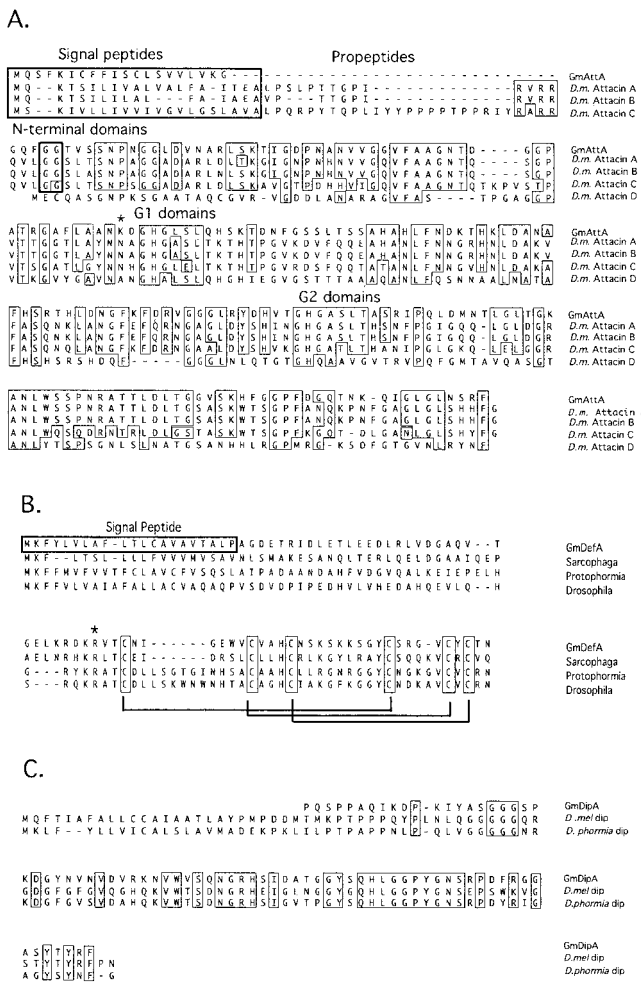

Fig. 1. Deduced amino acid sequence of antimicrobial genes characterized from tsetse and their comparative analysis to other genes. (*A*) *GmAttA* product compared with four putative attacin peptides described from *D. melanogaster*. The signal peptide, propeptide, N-terminal domain, and G1 and G2 domains are marked, and the residues that are conserved in GmAttA are boxed. * denotes the site of the intervening sequence in the genomic DNA separating the N-terminal domain from the G1 domain. *GmAttA*, AF368909; *D. m. attacinA*, P45884; *D. m. attacinB*, AAF71234; *D. m. attacinC*, AAG42833; *D. m. attacinD*, AAG42834. (*B*) *GmDefA* product aligned with other defensins, *GmDefA*. AF368907; *Sarcophaga*, P31529; *Protophormia*, P10891; and *Drosophila*, P36192. The putative signal peptide domain in *GmDefA* product is boxed. * denotes the R residue where the mature peptide begins. The six conserved cysteine residues are boxed, and the three putative disulphide bridges bonds are marked. (*C*) *GmDipA* partial product aligned with *diptericin* products. *GmDipA*, AF368906; *D. melanogaster*, AAB82532, and *Drosophila phormia*, P18684. The conserved residues are boxed.

domain. It exhibits high similarity to the *DipD* gene product of *Protophormia terraenovae* and the *DipB* product from *D. melanogaster* and, like other diptericins, has a short proline-rich N terminus domain (Fig. 1*C*). The putative GmDipA bears extensive amino acid sequence similarity to the C-terminal G-domain of *GmAttA*, indicating that the two families in *Glossina* may also share a common ancestor as is the case for the *attacin* and *diptericin* gene products of *Drosophila* (27).

Antimicrobial Gene Expression in Fat Body. Mock injections with PBS resulted in an increase in *attacin* and *defensin* transcription (Fig. 2). *E. coli* injection also resulted in increased levels of expression of *attacin* and *defensin* after 6 h, and they remained high, indicating that the response is pathogen specific and not

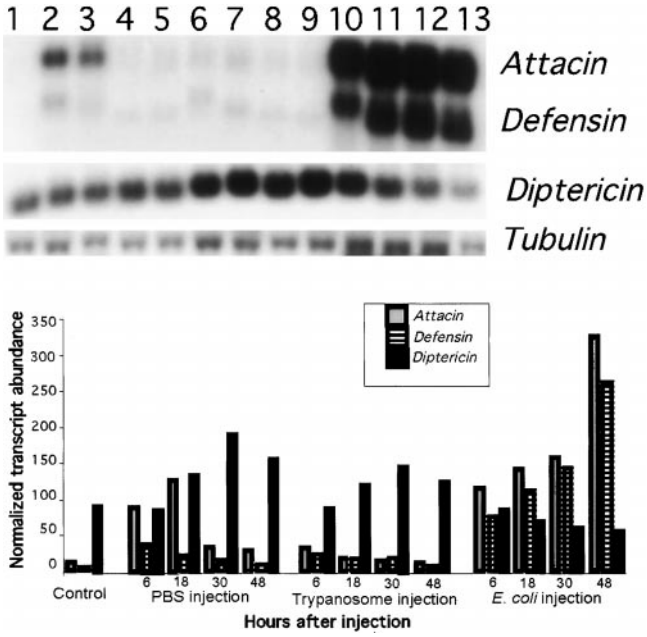

Fig. 2. Regulation of *attacin*, *defensin*, and *diptericin* gene expression in fat body after challenge by microinjection. Results are also schematically presented. Fat body RNA was analyzed from normal tissue (lane 1), 6, 18, 30, and 48 h after PBS injection (lanes 2–5, respectively), trypanosome injection (lanes 6–9, respectively), and *E. coli* injection (lanes 10–13, respectively). This is a representative example of three replicate experiments.

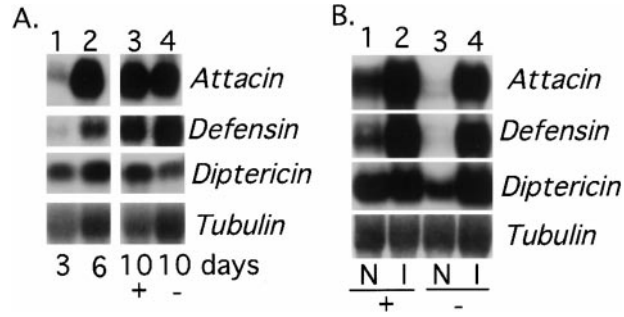

Fig. 4. Regulation of *attacin*, *defensin*, and *diptericin* expression in fat body during the course of parasite establishment and the immunocompetence of parasite-infected and infection-cured flies. (*A*) Northern analysis showing gene expression in fat body 3 and 6 days after a parasite infected bloodmeal (lanes 1 and 2, respectively) and after 10 days, when flies were scored as infected (+) or parasite infection cured (−) (lanes 3 and 4, respectively). (*B*) Gene expression in fat body from flies with (lane 1) and without (lane 3) gut parasite infections 20 days after receiving the infectious bloodmeal and their immunocompetence after challenge (lanes 2 and 4, respectively). +, parasite infected; −, parasite cured; N, naive; I, immune stimulated.

because of injury alone (Fig. 2). Injection of procyclic trypanosomes, however, resulted in lower levels of induction of *attacin* and *defensin* than achieved by PBS injection alone, and both gene expression levels dropped to uninjected fly levels by 30 h (Fig. 2). These results concur with those of a study showing tsetse hemolymph produced high antibacterial activity after *E. coli* injection but not after trypanosome injection (19).

Feeding procyclic parasites initially induced both *attacin* and *defensin* transcription, but both levels fell to control levels by 24 h (Fig. 3). In contrast, feeding bloodstream form trypanosomes did not raise *defensin* expression at all and induced *attacin* expression less than procyclics (Fig. 3). The presence of *E. coli* in the gut strongly induced both *attacin* and *defensin* transcription in fat body (Fig. 3), and the subsequent decrease in mRNA presumably reflects cell clearance from the gut. Analysis of unstimulated gut tissue indicated no expression of *attacin* but constitutive low-level expression of *defensin* (data not shown). Constitutive expression of *defensin* in gut tissue has also been noted in *Anopheles gambiae* (29) and in *Stomoxys calcitrans* (30).

Diptericin was found to be constitutively expressed in normal flies, and mock injection with *E. coli* resulted in only a modest induction over the basal level (Fig. 2). Injection or feeding of trypanosomes did not affect its expression level significantly (Figs. 2 and 3).

Antimicrobial Gene Expression During Parasite Establishment. Parasite establishment in the gut occurs in the first week after trypanosome uptake, during which time infected flies show increasing expression of both *attacin* and *defensin* genes (Fig. 4). By day 10, it is possible to determine microscopically which flies have established an infection and which have cleared the parasite. At 10 days, the expression of *attacin*, *defensin*, and *diptericin* was found to be high in infected and noninfected flies (Fig. 4*A*), but by 20 days, significantly less *attacin* and *defensin* transcript and, to a lesser extent, *diptericin* transcript was detected in flies that had eliminated parasite infections (Fig. 4*B*). In *Drosophila*

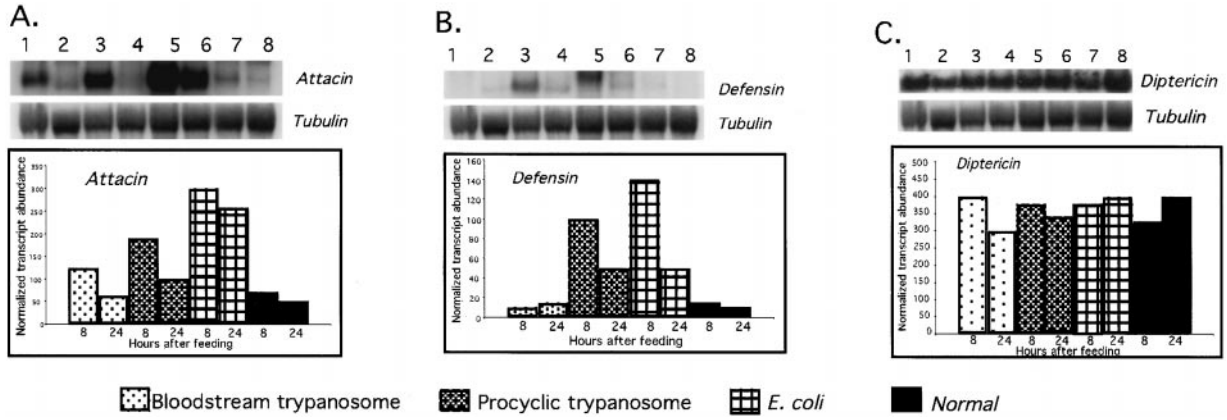

Fig. 3. Regulation of *attacin*, *defensin*, and *diptericin* expression in fat body after feeding pathogens in the bloodmeal. Northern blots were hybridized to *GmAttA* (*A*), *GmDefA* (*B*) and *GmDipA* (*C*) cDNAs, and results are also schematically presented. Fat body RNA was analyzed 8 and 24 h after bloodstream trypanosome feeding (lanes 1 and 2, respectively), 8 and 24 h after procyclic trypanosome feeding (lanes 3 and 4, respectively), 8 and 24 h after *E. coli* feeding (lanes 5 and 6, respectively), and 8 and 24 h after a normal bloodmeal (lanes 7 and 8, respectively). This is a representative example of three replicate experiments.

Table 1. Trypanosome infection prevalence in *G. m. morsitans* after immune activation with bacteria, LPS, or sterile PBS

Inoculation	Pooled, *N*	Prevalence (standard error), %	χ^2 analysis*
Control	145	49.7 (5.0)	—
E. coli	99	11.1 (5.1)	$P < 0.0001$
LPS	152	27.0 (2.5)	$P < 0.0001$
PBS	106	50.0 (9.6)	$P = 0.94$

Numbers represent pooled samples from replicates for each treatment, as explained in the text.

*The infection prevalence in the control groups served as the expected data against which other groups were tested.

with a midgut *Crithidia* infection, the expression of the antimicrobial peptides were also found to be up-regulated in fat body (31). To study the immunocompetence of parasite-infected tsetse, their response to immune challenge was monitored. The expression of all three genes increased in response to *E. coli* injection in infected flies, indicating that they were not immunosuppressed (Fig. 4*B*).

Antimicrobial Activity of Diptericin for Sodalis and Trypanosomes. The functional significance of the diptericin product for symbiont and trypanosome viability was determined by using the synthetic 82-mer diptericin (25). By using an *in vitro* growth assay with the synthetic diptericin, the IC_{50} value for procyclic trypanosomes was found to be at a concentration of 10 μM; Sodalis was 2 μM, whereas *E. coli* DH5α was 0.2 μM (data not shown). Sodalis was at least 10-fold less sensitive to the activity of diptericin than the related Gram-negative organism *E. coli*. The sensitivities of *E. coli* D22 and *E. coli* 1106 to the antibacterial activity of diptericin have similarly been shown to be less than 0.15 and 0.3 μM, respectively (25).

The Role of Innate Immune Responses During Parasite Establishment. A trypanosome-containing meal was given to teneral flies that were previously immune induced by either *E. coli* or LPS microinjection. Twenty days later, the prevalence of parasite infection in the control group was found to be 49.7%, whereas in the *E. coli*-stimulated group, it was about 11% and in the LPS group, 27% (*E. coli*, $P < 0.0001$; LPS, $P < 0.0001$) (Table 1). There was no significant difference between the control and PBS groups ($P = 0.94$). This dramatic decrease in trypanosome infection rate in immune-stimulated flies mirrors a similar finding for *Brugia malayi* transmission in *Aedes aegypti* (32) and *Plasmodium* transmission in *A. gambiae* (33).

Discussion

At the center of insect immune reactions is a diverse set of mechanisms, including phagocytosis, activation of proteolytic cascades, such as coagulation and melanization, and production of various antimicrobial peptides initiated in the major immune organ, the fat body (34). Because the immune stimuli included Gram-negative bacteria, a large number of clones selected were found to encode the antimicrobial peptides attacin, defensin, and diptericin. The defensin peptide has been shown to be active against Gram-positive bacteria in addition to having antiparasitic activity against the eukaryotic parasites *Plasmodium gallinaceum* (35) and *B. malayi* (32). The antibiotic spectrum of diptericin has been found to be similar to that of attacin and has been shown to be effective against Gram-negative bacteria in submicromolar quantity (25).

In normal tsetse, *attacin* expression was undetectable, whereas low-level expression of *defensin* and high levels of *diptericin* were observed. Although some constitutive expression of *defensin* has been previously noted, the constitutive expression of *diptericin* both in fat body and gut tissue is unprecedented in other insect systems in the absence of bacterial infections. In *Stomoxys,* infection of the gut with bacteria or fungal fractions did not result in increased expression of fat body responses (36). The results of Tzou *et al.* (37) suggest that *Drosophila* fat body immune peptide genes are also insensitive to midgut microbial infections. This difference is possibly explained by the fact that tsetse are obligate hematophages, feeding exclusively on sterile blood throughout life. In consequence, their gut epithelium will not be exposed to the regular microbial challenge received by *Stomoxys* and *Drosophila*, and they possibly lack the same means of constraining the infection to the gut lumen. To supplement their restricted diet, however, tsetse flies harbor two symbiotic microorganisms that represent evolutionarily ancient associations with the fly. One of the symbionts, *Sodalis*, is closely related to *E. coli* and lives intra- and extracellularly in midgut in addition to other somatic tissues, including the hemolymph. We suggest constitutive expression of *diptericin* may be because of these bacterial symbionts. Although the concentration of the diptericin peptide in tsetse hemolymph is not known, in our analysis, *Sodalis* was found to be 10 times more resistant to the activity of diptericin than its close relative *E. coli* and hence may not be significantly adversely affected by diptericin *in vivo* in tsetse. Alternatively, the tsetse fly may rely on the antibacterial activity of diptericin to control the numbers of its symbionts.

The immune response in tsetse is pathogen specific; it is capable of discriminating not only between bacteria and trypanosomes but also between the bloodstream form and procyclic trypanosomes. This specificity might reflect differences in the surface coats of the two forms of the parasite, the antigenically variable variant-surface glycoprotein covering the bloodstream forms versus the procyclin coat of the procyclic insect forms. Discrimination between pathogen groups is well known in *Drosophila*, where it is probably explained by the use of different receptor/signaling pathways. Thus expression of diptericin in *Drosophila* is regulated entirely through the imd/Relish pathway; defensin transcription involves imd/Relish and Toll/Dif, whereas attacin can be regulated by imd/Relish, Toll/Dif, and Toll/dorsal (38). Different receptor pathways may also explain the pathogen-specific immune response recorded here in tsetse flies.

During the normal course of infection, teneral flies would obtain bloodstream form parasites from feeds on infected animals and would exhibit a minimal initial response, but transcription of *attacin* and *defensin* would begin to build as these parasites transform to procyclics in midgut over the first 48 h. It appears that the continued presence of procyclic trypanosomes ensures that the levels of transcription of these genes remain high in flies with an established infection up to at least 20 days but eventually fall in flies that can eliminate infection. Induction of fat body immune peptides has also been reported from *Drosophila* in response to eukaryotic *Crithidia* gut infections (31). A possible explanation for systemic expression of immune peptides may be the synthesis of cytokine-like molecules in the midgut epithelium and their activation of fat body cells or, alternatively, diffusible substances such as NO could serve as chemical signals to distant organs. The migration of procyclics into the hemolymph from heavy parasite infections in the gut seems a less likely cause of up-regulation of fat body immunity genes, as we showed above that responses to microinjected procyclics were relatively modest and transient. If the high levels of antimicrobial transcripts in the fat body of infected flies result in the synthesis of active peptides, either the trypanosomes are resistant to them or, alternatively, they are protected from their harmful effects in their unique niche in the tsetse alimentary canal. Because expression-based studies detect transcriptional differences only, further characterization of immune peptides in hemolymph by additional criteria such as bioassays or matrix-assisted laser desorption

ionization–time of flight MS analysis will be necessary to understand the translational or posttranslational regulation of immune peptide synthesis in response to trypanosome infections.

Although the fat body is at the center of the immune response, effector molecules expressed in the midgut are increasingly being recognized as playing a role in immune reactions. In many, perhaps the majority of instances, the gut is the first barrier to the invading organism, hence the initial responses of gut epithelium are very crucial. Gut-specific gene products such as nitric oxide (39) or the phenoloxidase cascade (40) in mosquitoes or lectins in tsetse (41) have been implicated in the initial attrition of parasite numbers after uptake in the bloodmeal. Our analysis suggests that there may be molecular communication between immune responsive tissues in tsetse with the presence of pathogens in the gut, resulting in the activation of genes in the fat body. Our results also show that parasite establishment in the gut is drastically reduced when flies are immune stimulated by a pathogen systemically before an infectious bloodmeal. This decrease in pathogen viability may suggest a direct impact on the expression of immune molecules in the gut after systemic injury. Alternatively, overall metabolic changes, such as in iron metabolism or in systemic levels of free radicals, might make the environment adverse for trypanosome development after bacterial challenge. Although the nature of the molecule(s) responsible for trypanosome killing in immune stimulated tsetse gut is at present not known, similar subtractive analysis of genes expressed in teneral guts versus immune-stimulated teneral guts may aid in their further characterization.

One potential application of our findings is the development of novel tsetse-based control strategies to reduce trypanosomosis. Given the lack of an effective vaccine and affordable and efficacious drugs (42, 43) and sustainable vector control strategies (44), there is a definite need to augment existing disease control strategies or to develop new ecologically sound and effective approaches. Advances in recombinant DNA technologies have recently fueled the development of molecular genetic approaches for the control of vector-borne diseases. The most challenging application of this transgenic technology is to modulate vector competence by introducing and expressing foreign genes with antipathogenic properties that interfere with pathogen viability, development, or transmission (45). Recently, by using transgenic approaches, a defensin gene has been introduced and constitutively expressed in fat body in mosquitoes with similar goals (46). Although direct germ-line transformation in tsetse has been difficult given the viviparous nature of its reproductive biology, it has been possible to exploit its gut symbiont, Sodalis, to express foreign genes. It has also been possible to reintroduce the recombinant symbiont to tsetse (47, 48). Further characterization of gut molecules responsible for parasite killing early in the infection process and their constitutive expression in the symbionts in tsetse gut may render flies refractory for parasite transmission. Subsequent population replacement of susceptible flies with their engineered refractory counterparts might provide alternative control strategies in the long run. In the immediate future, this approach would augment existing control strategies such as the Sterile Insect Technique by providing release strains that are not capable of transmitting parasites and hence improve the efficacy of these technologies (45).

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